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STUDY ON THE INFLUENCE OF MODIFIED CHITOSAN ON THE PRESERVATION OF TIGER PRAWN *PENAEUS MONODON*

Rajesh Rajendran [1], *Paari K. A.* [2]

Address(es):
[1] State Key Laboratory of Catalysis (SKLC), Dalian Institute of Chemical Physics, Dalian, China.
[2] Department of Life science, CHRIST (Deemed to be University), Bangalore, Karnataka, India.

*Corresponding author: paaribiot@gmail.com

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ABSTRACT

Native chitosan, irradiated chitosan (5kGy and 10 kGy) and grafted chitosan was characterized and employed for the preservation of sea food *Penaeus monodon*. The grafting of metha acrylate onto natural native polymer chitosan was executed and the configuration and arrangement of covalent bonds in the grafted chitosan was demonstrated by performing, SEM, XRD, FTIR, TG and DSC analyses. The modified chitosan conferred antioxidant and antibacterial potential equivalent to or better than that of the unmodified chitosan in the stored *Penaeus monodon*. Modified chitosan treated *Penaeus monodon* produced less TBARS and TVB values than the control group.

Keywords: Irradiation, chitosan, antioxidant, lipid oxidation, frozen storage

INTRODUCTION

Foods products from sea are generally perishable that spoil sooner than other foods. Mounting alertness with reference to the safety of sea food commodities have recently led to copious developments in the field of fish preservation. Storage and processing of seafood products are constrained mainly due to the problems related to the progress of lipid oxidation and off-flavours associated rancidity **(Ali et al., 2019)**. In order to evade the usage of synthetic preservatives, natural substances that carry a 'green' image for preservation of sea food are now being developed in various studies. Chitosan, an amino polysaccharide constituting of glucosamine and *N*-acetyl glucosamine, has been used widely in food processing, medicine and biotechnology fields **(Majeti and Ravi, 2000; Harish Prashanth and Tharanathan, 2007)**. In the recent past, chitosan becomes an appealing molecule due to its antibacterial, film forming property, antioxidative and biodegradable ability **(Fan et al., 2009; Song et al., 2018; Kim and Thomas, 2007; Rhoades and Roller, 2007)**. Wide spectrum of antimicrobial activity for chitosan was reported against human pathogenic microorganisms **(Chen et al., 2010; Raafat and Sahl, 2009)**. Although a lot of studies have published the antimicrobial nature of this under used polymer, the precise mechanism for its antimicrobial activity remains vague. **Fang et al. (2010)** stated that the antimicrobial nature of chitosan is caused due to the distraction of the cell membrane of food borne pathogens. Shelf life of fishery products was extended by inhibiting the growth of *Pseudomonas* and *Shewanella* by the addition of chitosan **(Cao et al., 2009).** Radiation is now considered as a handy tool for modification of chitosan for enhancement of their biological activity **(Feng et al., 2008).** Irradiation was effective in enhancing the antimicrobial action of chitosan against *Escherichia coli* **(Kume et al., 2002).** However, a research carried out by **Lopez-Caballero et al. (2005)** demonstrated negative influence on microbial growth in the presence of powdered chitosan. Investigations on the radiation effects of chitosan showed pronounced increase of antimicrobial and antioxidant activity **(Czechowska-Biskup et al., 2005; Park et al., 2004).** Enhancement of antioxidant activity of chitosan by gamma irradiation at three different doses was stated by **Feng et al. (2008)**. Suitability of irradiated chitosan in inhibiting oxidative rancidity in meat sample has been reported **(Kanatt et al., 2004)**. Among the diverse modifications that are practicable, blending of synthetic polymer is also a handy mode where the properties of chitosan are personalized and a novel flexible material was developed **(Suyatma et al., 2004; Pourjavadi et al., 2003; Mahdavinia et al., 2004; Don et al., 2002).** PMMA was blended with chitosan for usage in various biomedical applications **(Radhakumary et al., 2005). El-Tahlawy et al. (2006)** investigated the changes in the properties of chitosan grafted by methyl acrylate and found that the grafted copolymer was having enhanced antiviral property compared to the unmodified chitosan. Synergistic consequence of chitosan covalently tagged with antimicrobials have been described **(Song et al., 2002; Chen et al., 1996)**. In this study, the antioxidant and antimicrobial activity of chitosan was enhanced using irradiation. The effect of modified chitosan derivatives were analysed during the preservation of *Penaeus monodon*.

MATERIALS AND METHODS

Chitosan was irradiated at doses of 5 and 10 kGy in the Gamma chamber facility available with the CIF, Pondicherry University. Cobalt 60 was the irradiation source. Irradiated chitosan was prepared following the protocol of **Kannat et al. (2004).** Treatment groups for *Penaeus monodon* preservation were divided as: control (without chitosan treatment): 5kGy irradiated chitosan treated group: 10kGy irradiated chitosan treated group: Blended chitosan treated group. *Penaeus monodon* was purchased from the local retail market. Immediately after purchase they were taken back to lab under sterile conditions.

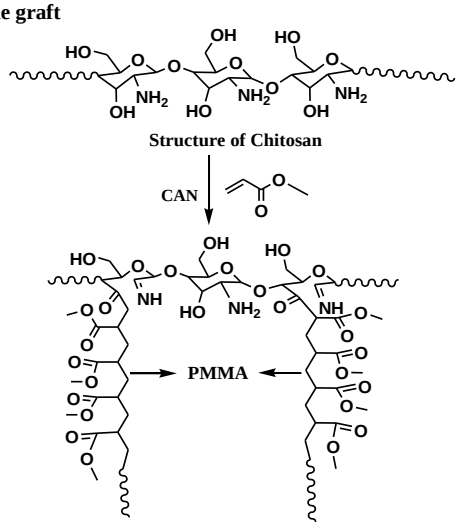
Synthesis of poly (methyl acrylate) polymer grafted chitosan (Chitosan-g-PMMA-Scheme- 1)

A 2g of 2% w/v chitosan solution was prepared in 250 ml of 1 % aqueous acetic acid. The reaction was carried out at 70 ^{0}C in nitrogen atmosphere. 0.1M Ceric Ammonium Nitrate in 10 ml of 1N nitric acid was then added. 4g MMA was added drop wise with continuous stirring. Sodium hydroxide solution was used to precipitate the product by continuous stirring for four hours and washed with distilled water several times. The homopolymer was extracted in a soxhelet from the grafted product using acetone as solvent. The percentage of grafting was calculated from Thermo gravimetric analysis (TGA)

Characterization

FT-IR spectra of chitosan and its graft was recorded in the range 4000–500 cm^{-1} using Nicolet Nexus 470 spectrometer. Chitosan and its graft copolymer was characterized using X-ray diffraction (XRD) and Scanning electron micrograph (SEM). The degradation process and thermal stability of chitosan and its graft copolymer was also analysed.

Scheme of the graft



Scheme-1 Structure of Chitosan-g-PMMA

DPPH radical Scavenging Activity

The potential to suppress the DPPH radical was monitored based on the method of **Blois (1958)**. Chitosan (0.1 ml) was mixed with 0.9 ml of DPPH (0.041 mM). After 60 min dark incubation, the reduction of the radical intensity was determined at 517 nm. The radical-quenching potential was calculated using the equation:

$$\%RSA = \left[\frac{(A_{DPPH} - A_s)}{A_{DPPH}}\right] \times 100$$

Reducing Power

The reducing power was determined following the method of **Oyaizu (1986)**. Chitosan (1 ml) was mixed with 0.2 M phosphate buffer (1 ml) and potassium ferricyanide (10 mg/ml) and incubated at 50°C. 1 ml of trichloroacetic acid (100 mg/ml) was added and centrifuged for 10 min. To the upper layer, 1 ml of H_2O and 0.1 ml of $FeCl_3$ (1.0 mg/ml) was added and the absorbance was read at 700 nm.

β-carotene bleaching assay

β-carotene bleaching assay was carried out based on the method of **Matthaus (2002)**. β-carotene was dissolved in 1 ml of chloroform. After removing chloroform, 40 mg of linoleic acid, 100 ml of distilled water and Tween 80 emulsifier were added. 0.2 ml of prepared chitosan extracts were added to this mixture and incubated at 50°C. The zero time absorbance and subsequent absorbance was recorded (470nm) at every 30 min intervals until the control sample had changed colour. Antioxidant potential was measured using the following equation

$$\text{Antioxidant activity} = \left[\frac{\beta - \text{carotene content after 120 min of assay}}{\text{Initial } \beta - \text{carotene content}}\right] \times 100$$

TBA assay

Oxidation of lipids was measured by the TBA assay as ascribed by **Ruberto and Baratta (1999)**. 0.05 g of sample (*Penaeus monodon*) was mixed with distilled water, 1.5 ml of 20 % acidic acid and 1.5 ml 0.8% of TBA in 1.1% SDS and heated to 100°C for 60 min. After cooling, 5 ml butan-1-ol was added. Samples were then centrifuged at 10000 rpm for 15 min. The absorbance of the upper layer was determined spectrophotometrically at 532 nm.

Determination of Total volatile base nitrogen (TVBN)

Microdiffusion method was employed to analyse total volatile base in *Penaeus monodon*. Briefly, 1 ml of H_2S0_4 was added into the inner chamber of the conway unit along with Tashiro's indicator. In the outer unit of apparatus, 1 ml of 20% $C_2HCl_3O_2$ extract and K_2CO_3 was added. The contents of the inner chamber of unit was titrated against NaOH (0.1 N) until a colour change was observed. A blank was carried out with 2% TCA.

Microbial count in raw *Penaeus monodon*

Total viable count was determined in the stored samples from control and all treatment groups. At time 0 and days 5, 10, 15, triplicate samples of each treatment were chosen for total plate count. For microbial analysis, tissue samples (10 gm) was aseptically homogenised using 90 ml of 0.85 % saline, for 1 min and was spread on the surface of plate count agar and incubated at 30° C for two days. Microbial load was expressed as log CFU/g

Statistical analysis

One-way analyses of variance were conducted to find out significant differences at $p<0.05$ using a SPSS package. Experiments were replicated thrice on dissimilar occasions with different fish samples.

RESULTS AND DISCUSSION

FT-IR spectra of pure chitosan and chitosan-g-PMMA reveal the process of successful grafting (Fig. 1A). In the spectrum of chitosan-g-Poly methyl methacrylate, the distinctive absorption bands around 3421, 1657 cm^{-1} can be seen. The broad absorption band at 3421cm^{-1} denote that -OH and -NH_2 groups are hydrogen bonded and absorption at 1657 cm^{-1} point out the amide linkage of chitosan backbone with external grafting polymer. In **figure 1A** (b) the bending frequency of -NH_2 (1457cm^{-1}) and -OH (1590cm^{-1}) peaks are shifted approximately by 15cm^{-1} compared to the spectra of native chitosan which assures the successful polymerization.

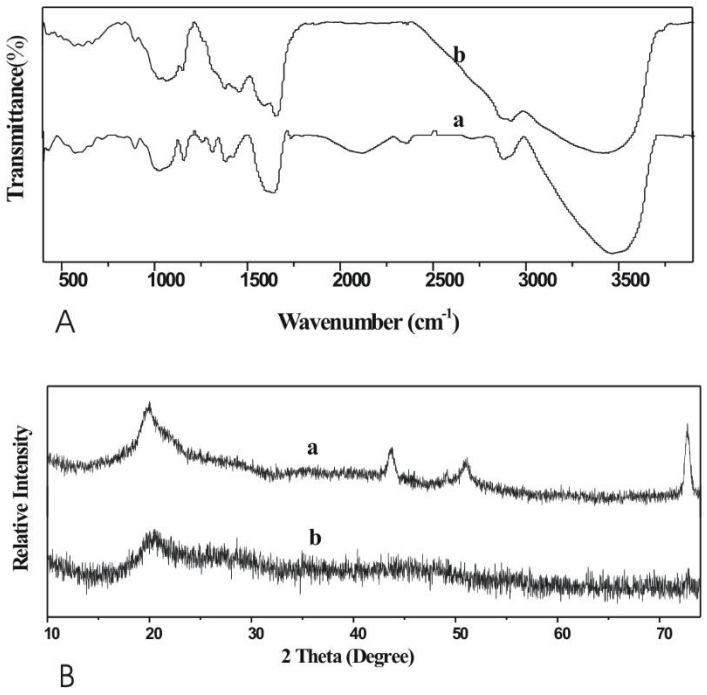


Figure 1 (1A) -FT-IR spectrumof chitosan (a) and chitosan-g-PMMA (b). (1B)-XRD spectrum of pure chitosan (a) and chitosan-g-PMMA (b)

The alteration of chitosan structure after polymerization was investigated by powder X-ray diffraction. The powder XRD marks, revealed successful grafting on the chitosan surface. Figure.1B (a, b) depicts the X-ray diffractograms acquired from pure chitosan and chitosan-g-PMMA. Four peaks of pure chitosan with high intensity at $2\theta = 20^0$, 44^0. 52^0 and 72^0 were observed and the obtained values matches the report of Joshi and Sinha *et al.* (2002) indicating the crystalline nature of chitosan. Subsequent to polymerization, three peaks on chitosan surface fully disappeared (**Figure 1B** (b)) which informs the loss of crystallinity in the grafted chitosan. A broad peak at 20^0 in grafted chitosan that signify the crystalline structure of chitosan is wholly transformed in to amorphous nature due to the polymerization. In the case of blended chitosan there was noteworthy variation in the intensity of distinctive peaks. The varied differences in the diffraction patterns between chitosan and grafted chitosan could be ascribed due to the adaptation in the arrangement of molecules in the crystal lattice.

Chitosan was observed as homogeneous particles in scanning electron microscopic images. Scanning electron image of native chitosan was used as the reference. The peripheral surfaces of grafts were accumulated in the form of globules as a consequence of the porosity of the graft which are the implications of graft copolymer. The SEM image also showed clustered configuration,

indicating the connections between chitosan molecules (**Figure 2**). Following graft copolymerization, irregular rod like and globular shapes were visibly observed.

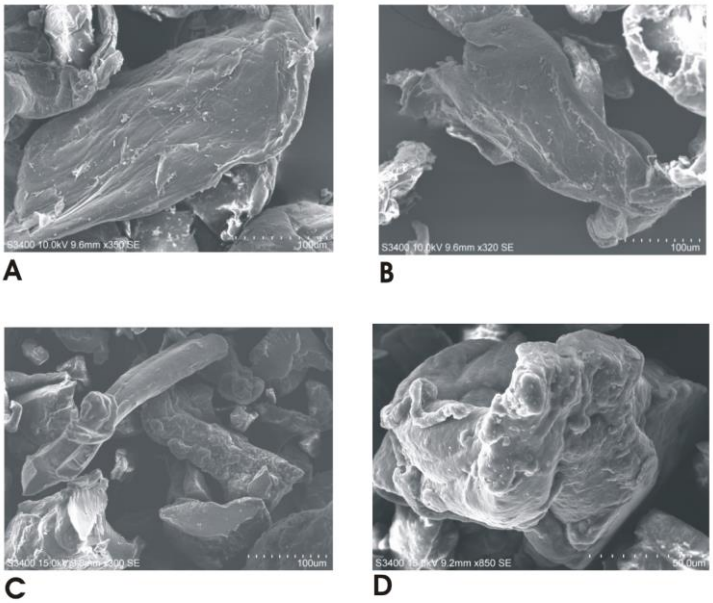


Figure 2 SEM image of (A,B) pure chitosan (C,D) chitosan-g-PMMA.

DSC shows the difference in Glass transition temperature (TG) between the pure chitosan and the grafted chitosan (**Fig. 3A**). Increase in molecular weight due to random polymerization and decline in glacious crystalline nature of grafted chitosan resulted in the decline of glass transition temperature. The blue shift in TG of grafts is due to external graft polymerization. Exothermic peak was found to be decreased in grafts due to increased molecular weight of external polymerization.

The degradation trend and thermal constancy of chitosan and chitosan grafts were evaluated through thermo-gravimetric analysis (TGA) experiments Weight loss in pure chitosan was 54.16% and starts at 245°C whereas weight loss starts at 208°C for PMMA grafted chitosan and the total weight loss for PMMA grafted chitosan was 49.63%. Decreased weight loss in case of grafted chitosan compared to native chitosan might be due to the reduction of saccharide units and increased polymerization.

Scavenging activity of modified chitosan:

Antioxidant activity of the chitosan determined by the bleaching of β-carotene was presented in fig. 4. Antioxidant activities were 62.33±3.39%, 67.3±5%, 81.2±2.34%, 87.9±2.2%, for unmodified chitosan, blended chitosan grafts, 5 kGy irradiated chitosan derivative, 10 kGy irradiated chitosan derivatives respectively. In the presence of customized chitosan, samples retained their colour for a longer time demonstrating the antioxidant potential of modified chitosan. The extent or potential of radical neutralisation varied with different modified chitosans. Modified chitosan reduced the extent of β-carotene destruction by neutralizing the linoleate free radical formed in the system. The high radical quenching property of chitosan might be due to reaction between the generated free radicals and the residual free amino groups **(Tamer et al., 2016)**.

The antioxidant activity in our study was found to be concomitant with the reducing power of native and modified chitosan samples. The highest reducing power was observed for irradiated chitosan derivatives compared to the unmodified and grafted chitosan. Difference in reducing power was also observed between the groups subjected to irradiation. A higher irradiation dose (10 kGy) exhibited higher radical scavenging activity and higher reducing power compared to chitosan irradiated at 5 kGy. The modified chitosan showed higher potential as electron donors to quench free radicals compared to the control (unmodified chitosan). Generally, radical reducing properties are coupled with the endurance of reductones, which split down the radical chain by donating a hydrogen atom (**Singh et al., 2002**). Reducing powers obtained were in the following order: unmodified chitosan > blended chitosan> 5kGy irradiated chitosan > 10kGy irradiated chitosan derivative.

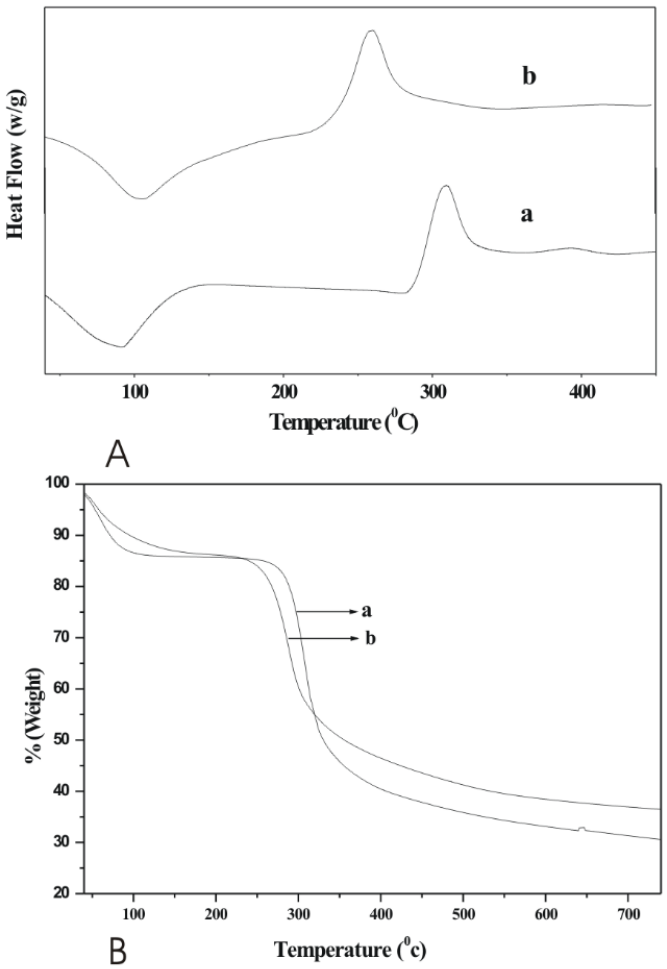


Figure 3 (3A)- DSC of (a) pure chitosan and (b) chitosan-g-PMMA. (3B)- Thermal gravimetric analysis (TGA) of (a) pure chitosan (b) chitosan-g-PMMA.

Figure 3B, shows thermograms of (a) pure chitosan and (b) chitosan-g-PMMA respectively. Pure chitosan show evidence of an endothermic peak at temperature around 91.84 ^{0}C, which ascribes to the water holding aptitude of chitosan by the -OH and free $–NH_2$ groups. Additionally, chitosan displays an exothermic peak at 304.60 ^{0}C informing decomposition of polysaccharide unit. In the case of figure 3B (b), endothermic peak appear at 101.92 ^{0}C and exothermic peak appear at 284.05 ^{0}C which explores the decreased decomposing peak of chitosan grafted polymer, indicating successful polymerization. Similarly, the glass transition temperature of the chitosan-g-PMMA progressively increased which confirms the decreased crystalline temperament of chitosan due to the grafting.

DPPH assay offers a rapid method for accessing the radical quenching potential. Proton radical scavenging potential was higher in 10 kGy irradiated chitosan derivative (84±2.5%) followed by 5 kGy irradiated chitosan derivative (77.35±2.57%), blended chitosan (65±1). Minimal DPPH scavenging activity was observed in unmodified natural chitosan (63.11±1.1%). It is evident from our result, that irradiation of chitosan enhance the antioxidant activity of chitosan. Also the enhancement or increase in antioxidant potential is dose dependent in case of irradiation (**Figure 4A**).

A similar study carried out by **Kannat et al. (2004)** revealed that irradiated chitosan exhibited enhanced antioxidant activity than the unirradiated chitosan. In another study carried out by **Feng et al. (2008)** chitosan irradiated at 20 kGy exhibited higher reductive capacity and better radical scavenging potential. Irradiation treatment has been reported to depolymerise chitosan, thus revealing the free amino groups which increase the DPPH radical quenching activity (**Kannat et al. 2004**).

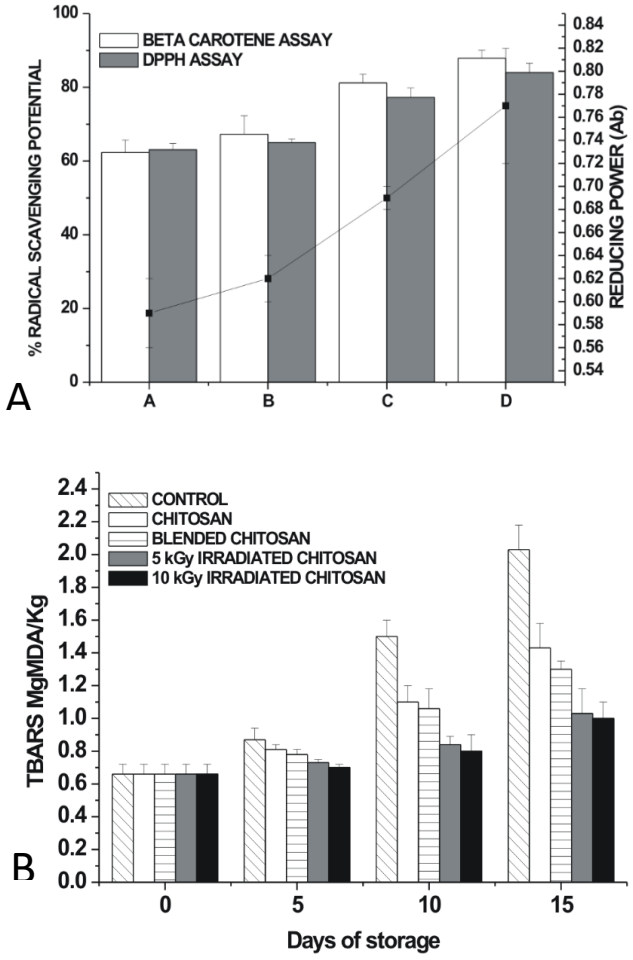


Figure 4A Antioxidant potential of chitosan extracts: A-chitosan. B-Blended chitosan C- 5 kGy irradiated chitosan. D- 10 kGy irradiated chitosan.
4B Evolution of TBARS values in *Penaeus monodon* (mg MDA Kg^{-1} of fish) during refrigerated storage.

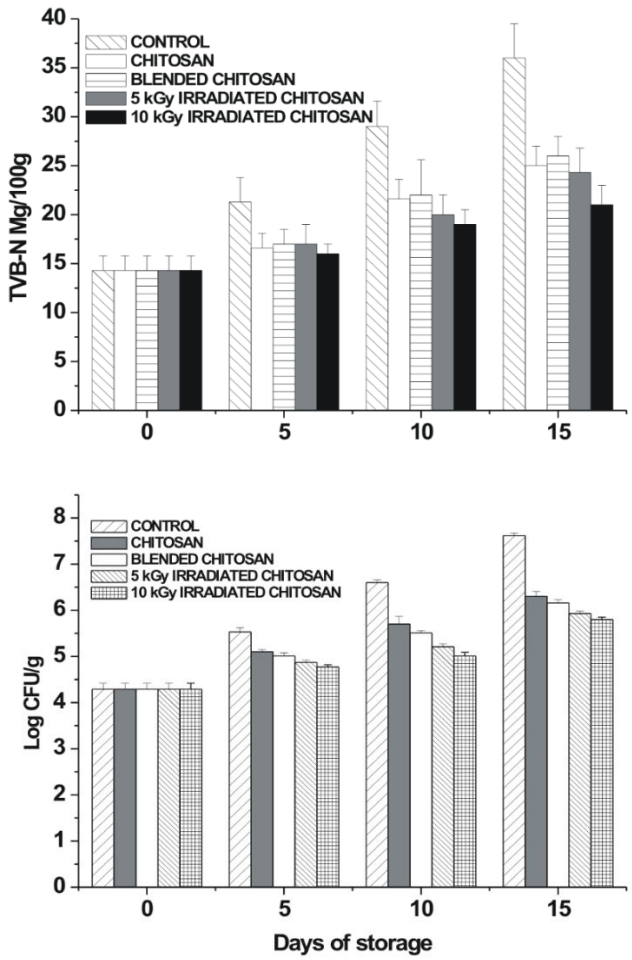


Figure 5A Evolution of TVB values in *Penaeus monodon* (TVB-N mg/100g) during refrigerated storage.
5B. Profiles of antimicrobial activity in *Penaeus monodon* treated with chitosan during refrigerated storage.

Effect of chitosan on t-bars evolution

This study demonstrated the effectiveness of native and modified chitosan as a natural anti-oxidant, when it was supplemented to the *Penaeus monodon*. Data generated using the TBARS assay in *Penaeus monodon* during refrigerated storage are presented in **Figure 4B**. Throughout the study period, *Penaeus monodon* treated with modified chitosan exhibited lower TBARS values indicative of higher antioxidant activity than the control (no treatment). Though *Penaeus monodon* treated with unmodified chitosan exhibited antioxidant activity better than control (without chitosan treatment), it was lower than the activity executed by the modified counterparts. The susceptibility of sea food products to endure rancidity mediated disorders during storage is chiefly due to the elevated amounts of unsaturated lipids. By the end of storage time, significant differences ($P>0.05$) were observed between the control (2.03±.15) and each of native chitosan, blended chitosan, 5 kGy , 10 kGy irradiated chitosan infused *Penaeus monodon*, which exhibited values of 1.43±.15, 1.3±.05, 1.03±.15, 1±.1 respectively. Throughout the study period, difference in TBARS value was not significant between the unmodified chitosan and blended chitosan but was significant with the samples treated with irradiated chitosan. Similarly, irradiated chitosan exhibited a better anti-oxidant activity in lamb meat than autoclaved chitosan **(Kannat et al., 2004)**. **Darmadji and Izumimoto (1994)** proposed that the anti-oxidative properties of chitosan are accountable for minimising the TBA values in minced beef.

Total volatile base

TVB-N contents augmented for all samples during the storage period, with the utmost values recorded for control samples followed by chitosan, blended chitosan, 5 kGy irradiated, 10 kGy irradiated chitosan (**Figure 5A**).

TVB values also intensified for chitosan treated samples during storage period. At the end of storage period, TVB value of control *P. monodon* was found to be 36±3.5which was higher than chitosan(26±2), blended chitosan treated (25±2), 5 kGy irradiated chitosan treated (24.3±2.5) 10 kGy chitosan treated (21±2)**.** No lag phase was detected for control samples during total volatile base generation; a rapid increase in TVB was evident from day-5. Stored control samples surpassed the acceptability margin (35 mg TVBN/100 g of fish) set by the European Union for total volatile base values of fish. Though limitation of TVB was not significant between the chitosan treatment groups, all chitosan treated samples, limited the generation of TVB throughout the storage period. A TVB value of 30 mg which is measured to be spoilage level for human consumption (Harpaz et al., 2003) was attained in control samples by day-10 whereas none of the chitosan treated groups reached this boundary. Reduction in TVB values by chitosan treatment was reported in fishery products (Jeon et al., 2006; Cao *et al*., 2009). Reduction of microbial load was evident in chitosan treated samples (Fig. 5B). Chitosan has been reported for its antimicrobial nature due to its polycationic character in various studies (Chen et al., 1998; Shin et al., 2001; Xie et al., 2002). Efficiency of irradiated chitosan on limiting the growth of *E. coli* was studied by **Matsuhashi and Kume (1997)**. Log reduction was high in irradiated chitosan treated samples (1.5 Log) compared to the unmodified chitosan (1.3 Log) treated samples (**Figure 5B**). Though Log reduction was not significant during early days of storage, marked reduction was noted during later days of storage between the control and treatment groups.

CONCLUSION

Our research discusses the application of radiation degraded (5 and 10 kGy) and grafted chitosan (CH-g-PMMA) for the preservation of sea food *Penaeus monodon.* Study confirmed that the modified chitosan exhibited better antioxidant and antibacterial potential in the stored *Penaeus monodon* which is evident from the less TBARS and TVB values and better sensory values. Designing a process or material for preserving and processing the sea food plays a crucial role in fulfilling the demands of the food industry.

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